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PRINCIPAL INVESTIGATOR: George A. Orr, Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine of Yeshiva University
Bronx, New York 10461

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George A. Orr, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Albert Einstein College of Medicine of Yeshiva University
Bronx, New York 10461

E-Mail: orr@aecom.yu.edu

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Alterations to microtubule dynamics, leading to a less stable polymer, may be a crucial determinant in the development of resistance towards Taxol, and other drugs with a binding site on the microtubule polymer. We propose that two potential mechanisms by which breast cancer cells could alter their microtubule dynamics are by (1) differential expression of the several α and ß tubulin isforms and (2) differential binding of endogenous regulators of microtubule assembly to the cytoskeleton as a result of posttranslational modifications to these tubulin isoforms.The overall goal of this proposal is to develop rapid and innovative protein-based technologies for both quantitating the α- and ß-tubulin isoform composition in drug-sensitive and -resistant human breast cell lines, and for characterizing the posttranslational modifications to these isoforms. It is only by thoroughly understanding Taxol resistance in human breast cancer cells that we will be able to develop ways to overcome Taxol resistance in breast cancer.

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Introduction

Alterations to microtubule dynamics, leading to a less stable polymer, may be an crucial determinant in the development of resistance towards Taxol, and other drugs with a binding site on the microtubule polymer. We propose that two potential mechanisms by which breast cancer cells could alter their microtubule dynamics are by (1) differential expression of the several α - and ß-tubulin isotypes and (2) differential binding of endogenous regulators of microtubule assembly to the cytoskeleton as a result of posttranslational modifications to these tubulin isoforms. Currently, there is no reliable method for quantitating the complete α/ß-tubulin protein isotype composition in human cells. Moreover, no precise structural information exists on the structural diversity of α - and ß-tubulin isoforms in human tumors, tissue, or cell lines. The overall goal of this proposal is to develop rapid and innovative protein-based technologies for both quantitating the α- and ß-tubulin isotype composition in drug-sensitive and -resistant human breast cell lines, and for characterizing the posttranslational modifications to these isoforms. Since the majority of the amino acid sequence differences, and also posttranslational modifications, occur within the C-terminal 20 amino acid residues of α/b-tubulin, we propose to develop a mass spectrometry-based methodology for the analysis of these C-terminal domains from human cell lines.

Body

Task 3/4 - To characterize and quantify the tubulin isotype composition in the parental drug-sensitive and resistant MDA-MB 231 and MCF-7 breast carcinoma cell lines.

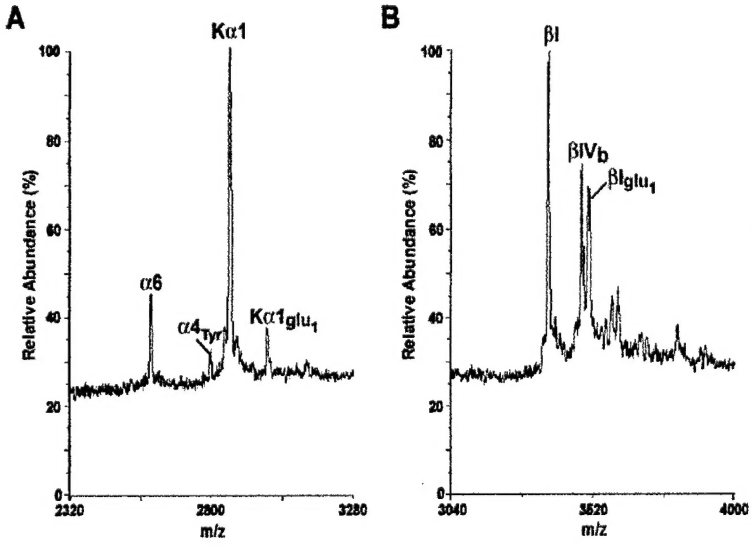

Fig 1. MALDI-TOF MS analysis of Taxol-purified Tubulins. Panel A, α-tubulins; panel B, β-tubulins.

1. Ion Suppression Effects in MALDI-TOF MS Analysis of C-terminal Tubulin Peptides - Ion suppression effects can be a significant problem in MALDI-TOF MS analysis. Our previous MALDI-TOF MS analysis of tubulin CNBr C-terminal peptides from total cell extracts may have under represented certain tubulin isotypes due to these ion suppression effects (Rao et al., 2001). In that study, the C-terminal peptides of ßII-, ßIII and ßIVa were not observed and the ßIVb peptide ion was minor compared to

ßI. Ions for minor tubulin species like βIII-tubulin or βIVb-tubulin could have been suppressed by the presence of the more highly abundant tubulin C-terminal peptides and/or because of differential ionization efficiencies of the various peptides. We reevaluated this approach by reducing the complexity of the sample prior to CNBr digestion (Verdier-Pinard et al., 2003). Tubulin was purified by the Taxol-based polymerization method from MDA-MB231 cell extracts followed by the separation of a-tubulin and ß-tubulin on SDS-PAGE gels prior to transfer to the nitrocellulose membrane. The region of the blot containing either α-tubulin or ß-tubulin was CNBr-digested and MALDI-TOF MS analysis was performed. The m/z peaks intensities corresponding to ßIVb and monoglutamylated ßI-tubulin C-terminal peptides were increased significantly compared to those obtained from unfractionated cell extracts (see Rao et al., 2001) . Very low levels of the C-terminal peptide of tyrosinated α4-tubulin were also detected. However, ßII-, ßIII and ßIVa tubulin C-terminal peptides were still undetectable (Verdier-Pinard et al., 2003).

It could be argued that the Taxol-dependent polymerization process selectively enriched for specific ß-tubulin isotypes. For this reason, immunoblot analysis was used to follow the isotype composition during the tubulin isolation step. Our data established that the isotype composition of the final Taxol-stabilized microtubule pellet was representative of the cellular tubulin composition (Verdier-Pinard et al., 2003).

2. Analysis of Tubulin Isotypes by combined Isoelectric Focusing and Mass Spectrometry - Due to the ion suppression effects noted above, it was necessary to modify our experimental protocol. The isolation of tubulin from cell extracts using the Taxol-driven polymerization coupled to high resolution isoelectric focusing enabled us to easily visualize and analyze the different tubulin isoforms present in cancer cell lines. The position of each tubulin band on the IPG strip was definitely assigned by performing in-gel trypsin digestion followed by MALDI-TOF MS analysis. After tryptic digestion of each excised band, mass analysis detected several isotype-specific tryptic peptides that confirmed the assignment of each band to an individual tubulin isotype class based on the sequence derived pI values (Verdier-Pinard et al., 2003). We confirmed the presence of the tubulin isotypes in the MDA-MB-231 and A549 cell lines identified previously by MALDI-TOF mass spectrometry analysis of C-terminal tubulin peptides (Rao et al., 2001). Because the C-terminal sequence obtained previously by MS/MS for α*-tubulin matches the C-terminal sequence of the recently described human α6-tubulin, and because in the present study a protein having the pI value predicted for human α6-tubulin was detected and confirmed as being α6-tubulin by peptide mapping, we consider them to be identical. An increase in βIII-tubulin expression was detected in resistant compared to sensitive cells. A minor βIII-tubulin species that corresponded most likely to monoglutamylated or phosphorylated βIII-tubulin was also detected.

The potential ion suppression effects are overcome in the present isoelectrofocusing/mass spectrometry approach since the total amount of each tubulin isotype is analyzed directly, giving a better appreciation of isotype ratios. Interestingly, the ratios of Kα1-tubulin: βI-tubulin and α6-tubulin: βIVb-tubulin are close to one in the cell lines examined so far. This trend suggests that there could be a specific pairing of a particular α-tubulin isotype with a particular β-tubulin isotype.

The most striking difference between our analysis of tubulin isotype expression at the protein level (Rao et al., 2001; Verdier-Pinard et al., 2003) and previous studies at the mRNA level (Kavallaris et al., 1997) is the absence of detectable βIVa- and βII-tubulin expression in both sensitive and resistant cell lines. Since our data established that tubulin was quantitatively recovered from cells by Taxol-driven polymerization (Verdier-Pinard et al., 2003), either there is an unknown bias in the RT-PCR results or there is a still unknown silencing mechanism of βII- and βIVa-tubulin mRNA translation. Increased βIII-tubulin expression levels have been repeatedly confirmed at the protein level using a variety of methods in cell lines resistant to Taxol (Nicoletti et al., 2001; Ranganathan et al., 1998a; Ranganathan et al., 1998b). Together with this present study, these observations strongly suggest that only βIII-tubulin levels correlate with sensitivity towards Taxol (Nicoletti et al., 2001; Ranganathan et al., 1998a; Ranganathan et al., 1998b) and that the regulation of β-tubulin expression is even more complex than the autoregulatory mechanism described by Cleveland and colleagues (Cleveland and Sullivan, 1985).

3. Evaluation of Tubulin isotype-specific Antibodies - Our ability to resolve and identify each tubulin isotype on isoelectric focusing gels allowed us to evaluate the specificity of a panel of tubulin isotype-specific antibodies (Verdier-Pinard et al., 2003). The anti-β-tubulin isotype monoclonal antibodies produced and characterized by Dr Ludueña and co-workers (Banerjee et al., 1990; Luduena, 1998) represent valuable tools that have helped to unravel the functional significance of the different β-tubulin isotypes. No cross-reactivity of the anti-βI-, -βIII-, and -βIV- tubulin antibodies was observed in the present study. This is contrary to what has been published for non-denaturing immunoaffinity isolation of α/β-tubulin heterodimers enriched in βI-, βII-, βIII- or βIV-tubulin isotypes (Banerjee et al., 1992; Luduena, 1998). Therefore, the presence of contaminating β-tubulin isotypes of a different class in these fractions is more likely due to heterodimer-heterodimer interactions in residual small oligomers than to cross-reactivity of these antibodies with multiple isotypes. However, extensive cross-reactivity of the anti-βII-tubulin antibody with βI-tubulin was confirmed.

Key Research Accomplishments

- Developed a combined high resolution isoelectric focusing/mass spectrometry-based method for the analysis of tubulin isotypes and mutations from Taxol-resistant human cell lines.
- The absence of ßII and ßIVa tubulins in the MDA-MB-231 breast carcinoma cell line has been confirmed by this method.
- Increased expression of ßIII-tubulin in a Taxol-resistant MDA-MB-231 cell line has been detected suggesting that this isotype may be a unique marker of resistance.
- The method was used to established the specifictity of the anti-ß1, ßII, ßIII and ßIV monoclonal antibodies.

Reportable Outcomes

Verdier-Pinard, P., Wang, F., Martello, L.A., Horwitz, S.B. And **Orr, G.A** (2002) Mass Spectrometry-based Analysis of Tubulin Isoforms in Taxol® Sensitive- and Resistant-Human Cancer Cell Lines. Abstract presented at the first annual HUPO meeting, Versaille, France November 2002.

Verdier-Pinard, P., Wang, F., Martello, L.A., **Orr, G.A**. and Horwitz, S.B. (2003) Analysis of Tubulin Isotypes and Mutations from Taxol-resistant Cells by Combined Isoelectrofocusing and Mass Spectrometry. *Biochemistry* **42**, 5349-5357.

Orr, G.A., Verdier-Pinard, P., McDaid, H., He, L. and Horwitz, S.B. (2003) Mechanisms of Taxol resistance related to microtubules. *Oncogene*, **in press**.

Conclusions

An emerging theme in studies of Taxol resistance is that alterations to microtubule dynamics may be a crucial factor in the development of resistance. Microtubule assembly can be potentially altered by differential expression and/or posttranslational modifications to tubulin isotypes. Although PCR- and microarray-based technologies can be used to measure mRNA expression levels for tubulins, there is a need for the development of rapid and sensitive protein-based methods for determining the tubulin protein isotype composition, and characterizing posttranslational modifications to these isotypes, in drug-sensitive and resistant cell lines. The data presented in this progress report demonstrate that high-resolution isoelectric focusing

combined with mass spectrometry is a powerful approach for the analysis of tubulins expressed in drug-sensitive and resistant cell lines. Importantly, our studies confirm an increased expression of ßIII-tubulin in resistant cells indicating that this tubulin isotype is a unique marker of resistance. The information gained from these experiments will provide a greater understanding of the modifications that occur to microtubules when human breast tumors become resistant to Taxol.

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Rao S, Aberg F, Nieves E, Band Horwitz S and Orr GA (2001) Identification by mass spectrometry of a new alpha-tubulin isotype expressed in human breast and lung carcinoma cell lines. *Biochemistry* **40**:2096-103.

Verdier-Pinard P, Wang F, Martello L, Burd B, Orr GA and Horwitz SB (2003) Analysis of tubulin isotypes and mutations from taxol-resistant cells by combined isoelectrofocusing and mass spectrometry. *Biochemistry* **42**:5349-57.

Analysis of Tubulin Isotypes and Mutations from Taxol-Resistant Cells by Combined Isoelectrofocusing and Mass Spectrometry†

Pascal Verdier-Pinard,‡ Fang Wang,§ Laura Martello,‡,|| Berta Burd,‡ George A. Orr,‡ and Susan Band Horwitz*,‡

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461, and Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: Six human α-tubulin and seven human β-tubulin isotypes, each of which can undergo posttranslational modifications, have been detected by the reverse transcriptase−polymerase chain reaction. This repertoire of tubulin isotypes plays a role in development and in the building of specialized microtubule-based structures. In cell lines, the relationship between resistance to microtubule-interacting drugs and altered tubulin isotype expression profiles is often established by quantitation of cDNA and/or Western blot analysis. Tubulin mutations in major isotypes are detected by sequencing cDNA, but more analysis of expression of tubulin mutations at the protein level, to assess their role in drug resistance, is needed. We utilized a Taxol-based purification and high-resolution isoelectrofocusing combined with a mass spectrometry-based analysis of tubulin. This approach has allowed the separation and relative quantitation of tubulin isotypes having a difference in isoelectric point values of 0.01, without the need for two-dimensional gel electrophoresis. The specificity of tubulin isotype antibodies also has been established. In cell lines resistant to microtubule-stabilizing drugs that express heterozygous tubulin mutations, the relative amount of mutant tubulin expression has been determined. In these cell lines, the absence of βII- and βIVa-tubulin has been demonstrated, and an increased level of expression of βIII-tubulin in resistant cells has been confirmed, indicating that this tubulin isotype is a unique marker of resistance.

Studies on the microtubule cytoskeleton reveal a spectrum of activities from structural to mechanical to signal transduction processes (*1*). Mitosis is a microtubule-dependent process, and inhibition of mitotic spindle function represents the mode of action of major anticancer drugs, i.e., the taxanes and vinca alkaloids. Microtubules are built upon the assembly of α/β-tubulin heterodimers. In human tissues, the expression of six α- and seven β-tubulin isotypes has been detected (Table 1). Additionally, tubulin undergoes posttranslational modifications, and complex patterns of tubulin isoform expression can be observed, especially in the central nervous system (*2*).

The existence of multiple tubulin species raises the question of the physiological relevance of such diversity (*3*). The greatest sequence divergence among tubulin isotypes resides in their C-termini (Table 1), sites that are exposed at the surface of microtubules and interact with microtubule-associated proteins and undergo posttranslational modifications. Specific tubulin isotypes are constituents of specialized microtubules (*2*). Moreover, alterations in tubulin isotype expression profiles and tubulin mutations have been reported in cell lines resistant to antimitotic agents (*4*−*8*).

The expression of a given tubulin isotype in a cell line or tissue is usually detected by quantification and sequencing of RT-PCR[1] products. Using this method, mutations in tubulin have been detected in cell lines resistant to Taxol and the epothilones (*5*−*8*). Despite the convenience of this approach, it does not give direct access to the corresponding protein expression levels. This is particularly relevant to β-tubulin mRNA because it appears to be autoregulated by the level of the free β-tubulin pool in cells (*9*). Similarly, heterozygous transcriptional expression of a tubulin mutation does not indicate that both wild-type and mutant tubulin proteins are expressed and, if so, at what ratio. Antibodies directed against the C-terminus of each tubulin isotype and against some of their posttranslational modifications provide useful tools for evaluating their respective levels of expression. However, possible cross-reactivity of these antibodies against different tubulin isotypes may introduce some quantitation bias, because of the lack of resolution of the different tubulin isoforms by SDS−PAGE.

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* To whom correspondence should be addressed. Phone: (718) 430-2163. Fax: (718) 430-8959. E-mail: shorwitz@aecom.yu.edu.

‡ Department of Molecular Pharmacology.

§ Laboratory for Macromolecular Analysis and Proteomics.

|| Present address: Department of Pathology, New York University School of Medicine, New York, NY 10016.

[1] Abbreviations: RT-PCR, reverse transcriptase−polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; DTT, dithiothreitol; IPG, immobilized pH gradient; IEF, isoelectric focusing; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; LC−MS, liquid chromatography coupled to mass spectrometry; 2D, two-dimensional; pI, isoelectric point.

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Table 1: Human Tubulin Isotypes

Isotype (accession #)	Human gene	C-terminus sequence[a]	Tissue expression	pI[b]
α-TUBULINS				
1 (I77403)	TUBA1/k-α1	MAALEKDYEEVGVDSVEGEGEEEGEEY	Widely expressed	4.94
1 (CAA25855)	TUBA3/b-α1	MAALEKDYEEVGVHSVEGEGEEEGEEY	Mainly in brain	5.02
3 (Q13748)	TUBA2	LAALEKDYEEVGVDSVEAEAEEEGEEY	Testis-specific	4.98
4 (A25873)	TUBA4	MAALEKDYEEVGIDSYEDEDEGEE	Brain, muscle	4.95
6 (Q9BQE3)	TUBA6	MAALEKDYEEVGADSADGEDEGEEY	Widely expressed	4.96
8 (Q9NY65)	TUBA8	LAALEKDYEEVGTDSFEEENEGEEF	Heart, muscle, testis	4.94
β-TUBULINS				
I (AAD33873)	HM40/TUBB	YQDATAEEEEDFGEEAEEEA	Constitutive	4.78
II (AAH01352)	Hβ9/TUBB2	YQDATADEQGEFEEEEGEDEA	Major neuronal, lung	4.78
III (AAH00748)	Hβ4/TUBB4	YQDATAEEEGEMYEDDEEESEAQGPK	Minor neuronal, testis	4.83
IVa[c] (P04350)	Hβ5/TUBB5	YQDATAEQGEFEEEAFEEVA	Brain specific	4.81
(NP_006078)		YQDATAEEGEFEEEAEEEVA		4.78
IVb (P05217)	Hβ2	YQDATAEEEGEFEEEAEEEVA	Major testis	4.79
V (NP_115914)	5-beta/Beta V	YQDATANDGEEAFEDEEEEIDG	Uterine adenocarcinoma	4.77
VI (NP_110400)	Hβ1/TUBB1	FQDAKAVLEDEEVTEEAEMEPEDKGH	Blood	5.05

[a] Amino acids differing from isotype 1 (Kα1) for α-tubulins or from isotype I (HM40/TUBB) for β-tubulins are highlighted in black. [b] The isoelectric points were calculated on the basis of the tubulin primary sequences found in the NCBI protein database (accession numbers given in first column) using the ExPaSy Compute pI/MW tool. [c] Two βIVa-tubulin sequences with distinct C-termini were found in the NCBI protein database. The top C-terminus sequence was found in human brain, and the bottom sequence was found in a human oligodendroglioma and in mouse brain.

We sought to perform both qualitative and quantitative analysis of tubulin content in cancer cell lines where tubulin represents ~3−4% of the total protein (*10*−*12*). Here we present a method based on Taxol-driven polymerization of total soluble tubulin from cell lysates. The resulting Taxol-stabilized microtubule pellets were analyzed by high-resolution isoelectrofocusing on immobilized pH gradient strips. Mass spectrometry analysis of in-gel tryptic digests definitively assigned each major tubulin species to a tubulin isotype class. Western blotting of these strips further established the specificity of tubulin isotype-specific antibodies. This methodology allowed us to evaluate the percentage of each tubulin isotype in cancer cell lines and to detect the expression of charge-altering mutations in tubulin from both Taxol- and epothilone-resistant cell lines. Additionally, the apparent absence of detectable βII- and βIVa-tubulin protein in cell lines where these isotypes were detected at the mRNA level demonstrated that RT-PCR-based analysis of tubulin isotype expression has to be interpreted cautiously. The increase in the level of βIII-tubulin expression in cell lines resistant to microtubule-interacting drugs may represent a significant tubulin isotype-related marker.

MATERIALS AND METHODS

Chemicals. Taxol was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD), dissolved in sterile dimethyl sulfoxide, and stored at −20 °C. Trypsin was obtained from Promega (Madison, WI). All other chemicals were obtained from Sigma (St. Louis, MO) except where noted.

Cell Culture. The human A549 lung, MDA-MB-231 breast, HeLa cervical carcinoma, and CA46 Burkitt lymphoma cell lines were maintained as described previously (*10*, *13*, *14*).

Isolation of Tubulin from Cell Lines. Subconfluent cultures of cells from seven to ten 100 mm dishes were washed once with PBS and scraped in 1 mL of PBS. For CA46 cells, 250 mL of suspension culture at a cell density of 10^6 cells/mL was centrifuged and the cell pellets were washed with PBS. Pellets of 0.3−0.4 mL of packed cells were obtained. One and one-half volumes of MME buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (pH 6.9), 1 mM $MgCl_2$, and 1 mM EGTA] was used to resuspend the cell pellets. The resulting cell suspensions were frozen in liquid nitrogen. Upon use, cell suspensions were rapidly thawed, and $^1/_{10}$ volume of a 10-fold stock solution of protease inhibitor cocktail (Boehringer Mannheim) in MME buffer and 1 mM DTT were added. Cell suspensions were sonicated with a microtip probe (Ultrasonics) seven times for 30 s with 30 s rest intervals on melted ice. Cell lysates were centrifuged at 120000*g* (Beckman TLA100.3 rotor) for 1 h at 4 °C. Cytosolic supernatants (SI) were transferred to new tubes, and the DNA and cell debris pellets (PI) were discarded.

Taxol-stabilized microtubule pellets were isolated by following the method of Vallee (*15*). In brief, cytosolic supernatants were incubated at 37 °C in the presence of 10

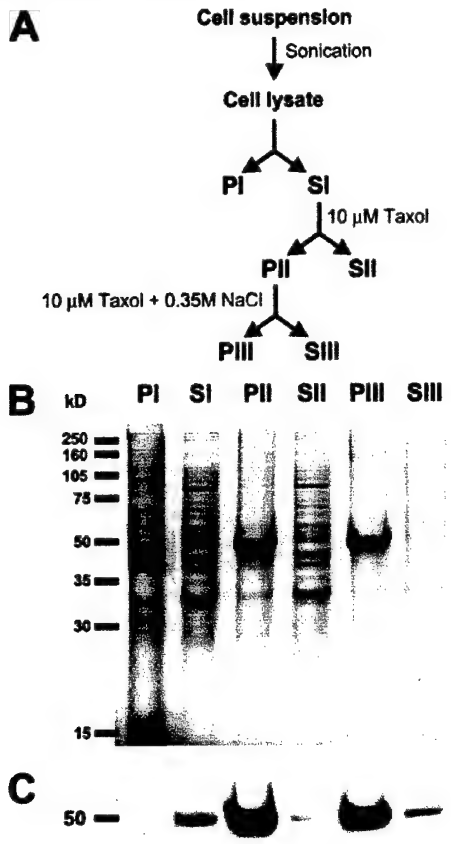

FIGURE 1: Taxol-based purification of tubulin from A549 cells. (A) Tubulin purification scheme (see Materials and Methods for details): PI, DNA and cell debris pellet; SI, cytosolic extract; PII, Taxol-stabilized microtubule pellet; SII, supernatant II; PIII, washed Taxol-stabilized microtubule pellet; SIII, supernatant III. (B) The different fractions were analyzed by SDS−PAGE (10% acrylamide gel, MW markers indicated on the left), and the gel was stained with Coomassie blue. (C) After SDS−PAGE of the same fractions, proteins were transferred to a nitrocellulose membrane that was probed with a pan α-tubulin antibody.

μM Taxol and 1 mM GTP for 20 min. Reaction mixtures were layered on a 0.1 mL cushion containing 5% sucrose in MME, 10 μM Taxol, and 1 mM GTP. Samples were centrifuged at 80000*g* (Beckman TLA100.3 rotor) for 30 min at 37 °C. Microtubule pellets (PII) were washed and resuspended in 0.1 mL of MME buffer containing 0.35 M NaCl and 10 μM Taxol. After centrifugation at 80000*g* for 30 min at 37 °C, microtubule pellets (PIII) were frozen on dry ice and kept at −70 °C until they were used. The Taxol-based purification of tubulin from A549 cells was monitored by SDS−PAGE and Western blot analyses (Figure 1) using either DM1A, a pan α-tubulin antibody, or DM1B, a pan β-tubulin antibody (Biogenex, San Ramon, CA), and pure bovine brain tubulin as a standard (Cytoskeleton Inc., Denver, CO).

Isoelectric Focusing. Microtubule pellets (containing approximately 100−200 μg of protein) were resuspended in 350 μL of solubilization buffer {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.5% Triton X-100, 0.5% ampholyte-containing buffer (pH 4.5−5.5), 20 mM DTT, and bromophenol blue}, loaded onto 18 cm IPG strips at pH 4.5−5.5 (Amersham Pharmacia Biotech), and run on an IPGphor IEF system for a total of 70 000 Vh. IPG strips were then stored at −20 °C. Prior to use, IPG strips were incubated in equilibration buffer [125 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, and 1% SDS] for 2 × 20 min each, and then incubated in IPG strip transfer buffer [25 mM Tris-HCl (pH 8.3) and 192 mM glycine] for 20 min.

Coomassie Blue Staining. IPG strips were fixed in a 50% methanol/20% trichloroacetic acid mixture for 30 min, stained with 0.1% R250 Coomassie blue in a 50% methanol/10% acetic acid mixture, destained in a 35% methanol/10% acetic acid mixture, incubated for 30 min in 2% glycerol, layered on filter paper with the plastic backing facing the paper, covered with a piece of transparent plastic sheet, and air-dried prior to scanning. IPG strips were scanned and bands quantitated using Image Quant (Amersham).

Western Blotting of IPG Strips. IPG strips were electrotransferred onto nitrocellulose membranes with the gel facing the membrane (without removing the plastic backing from the strip) and transferred for approximately 20 h at 100 mA. Only a partial transfer of proteins was achieved, and after being transferred, strips were directly fixed and stained with Coomassie blue stain (see above) so that the blots and gel could be aligned.

The β-tubulin isotype content was examined using a panel of mouse monoclonal antibodies directed against the C-terminus of βI-, βII-, βIII-, and βIV-tubulins originally produced by R. F. Ludueña and co-workers. The anti-βI-tubulin antibody (generous gift from R. F. Ludueña) was used at a 1:10000 dilution; the anti βII-, βIII-, and βIV-tubulin antibodies (Biogenex) were used at a 1:250 dilution, and a goat anti-mouse HRP-linked IgG was used as the secondary antibody (1:2000 dilution, Transduction Laboratories, San Jose, CA).

Membranes were probed for α-tubulin isotypes with an anti-Kα1-tubulin rabbit polyclonal antibody (SRa1) at a 1:20000 dilution or an anti-α6-tubulin rabbit polyclonal antibody (SRa6) at a 1:5000 dilution. A donkey anti-rabbit HRP-linked IgG was used as the secondary antibody (1:2000 dilution, Amersham). Antibodies SRa1 and SRa6 were prepared using synthetic C-terminus peptides GVDSVEGEGEE and GADSADGEDEG as antigens, respectively. A Cys residue was added at the N-terminus of these peptides for their conjugation to maleimide-activated keyhole limpet (Pierce, Rockford, IL). The conjugated peptides were injected into rabbits, and bleeds were analyzed by an enzyme-linked immunosorbent assay using the same Kα1- and α6-tubulin C-terminus peptides conjugated to maleimide-activated bovine serum albumin (Pierce). All blots were visualized using an enhanced chemiluminescence detection system (Amersham).

The only major non-tubulin proteins in the salt-extracted microtubule pellets (PIII) were γ- and β-actin, whose pIs are more basic than those of the tubulins. Blots were probed with a monoclonal anti-actin antibody (1:1000 dilution, clone AC-40, Sigma), allowing precise alignment of the different blots and the corresponding transferred Coomassie blue-stained gel. Membranes were first probed with a tubulin isotype-specific antibody and then with the anti-actin antibody, and finally, they were stripped before another tubulin isotype-specific antibody was used.

IPG Strip in-Gel Trypsin Digestion. IPG strips were stained with imidazole and zinc salts according to the

procedure of Castellanos-Serra et al. (*16*). Tubulin binds zinc, and positively stained tubulin bands appeared. The bands were cut with the plastic backing using scissors, washed with 1 mL of water (2 × 5 min, 1 × 10 min), and dehydrated in 90% aqueous acetonitrile (0.5 mL, 2 min). After centrifugal evaporation of residual acetonitrile, 25 μL of 12.5 ng/μL trypsin in 20 mM ammonium bicarbonate buffer (pH 8.3) containing 80 mM glycine was added to each tube. Trypsin digestion was performed at 37 °C overnight with constant shaking. The supernatant was desalted on a Millipore C18 Ziptip, and the tryptic peptides were eluted from the ZipTip with 4.0 μL of a 50% acetonitrile/H_2O mixture containing 0.1% trifluoroacetic acid.

Protein Identification. One microliter of a tryptic peptide mixture was mixed with 1 μL of a saturated α-cyano-4-hydroxycinnamic acid solution in a 50% acetonitrile solution containing 0.1% TFA, and 1 μL of the resulting solution was deposited on a clean mass spectrometer probe surface. Mass spectra were recorded in the positive or negative mode on a Voyager-DE STR MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA) equipped with a 2.0 m flight tube and a 337 nm nitrogen laser. Protein identification was accomplished through a database search (Swiss-Prot and NCBI) using MS-Fit and ProFound programs (*Homo sapiens/Mus musculus*, mass measurement error within 1 Da, partially oxidized methionine, average and/or monoisotopic masses, and two maximum miscleavages). The isoelectric point for each tubulin was calculated using the Compute MW/p*I* tool from the ExPaSy Web site.

RESULTS

Taxol-Based Purification of Tubulin from Cell Lines. Taxol microtubule pellets were isolated from cell lines using the method of Vallee (*15*). A representative preparation of tubulin from A549 cells is presented in Figure 1. There was no detectable tubulin in the first pellet (PI) containing cell debris and DNA, indicating that essentially all tubulin in the cell lysate (SI) was present in a soluble form. Tubulin polymerization by Taxol was quantitative as 98% of the tubulin present in SI was recovered in the Taxol-stabilized microtubule pellet (PII). The Taxol-stabilized microtubule pellet was washed with NaCl to release microtubule-associated proteins (MAPs). The resulting pellet (PIII) was highly enriched in tubulin, but a significant presence of tubulin was observed in SIII. Nevertheless, this loss from PIII was random regarding tubulin isotypes (data not shown) and most likely represented denatured tubulin. Therefore, the final microtubule pellet, PIII, was representative of cellular tubulin composition. We estimated that ~0.3 mg of tubulin was obtained from each preparation. Tubulin represented ~2% of total protein and approximately 5% of total soluble proteins in A549 cells.

Separation of Tubulin Isotypes by Isoelectric Focusing. IPG plates (pH 4.5−5.4) and IPG strips (pH 4−7) have been previously used to separate tubulin isoforms (*17*−*19*), but as indicated in Table 1, differences in pI values between two isotypes can be as small as 0.01. Only narrow-range IPG gels (pH 4.5−5.5) can resolve tubulin isotypes with such small differences in pI values. The length of the IPG gel and the shape of the pH gradient are also factors that increase the maximum separation of proteins. Therefore, we used 18 cm IPG strips with a linear pH gradient from 4.5 to 5.5, on which tubulin bands having a pI difference of only 0.01 could be separated from each other by 2 mm. To visualize tubulin isotypes, tubulin in cell extracts was purified and concentrated by Taxol-driven polymerization. This approach allowed us to use Coomassie blue staining of the IPG strips. On the basis of our previous studies in A549 non-small cell lung carcinoma cells (*13*, *14*), we expected to find the following tubulin isotypes from acidic to basic pI: βI-, βIVb-, βIII-, Kα1-, and α6-tubulin. Lane 1 in Figure 2B shows that we effectively separated four major protein bands contained in Taxol-stabilized microtubules from A549 cells. Two bands were in the pI range expected for most β-tubulins, and two bands were in the pI range for α-tubulins (Table 1 and Figure 2A). In A549-T12 cells, an A549 Taxol-resistant cell line containing a Ser to Arg substitution at residue 379 in Kα1-tubulin, an additional major band in the pI range for α-tubulins and a minor band with an intermediate pI between those of α- and β-tubulins were observed. The differences in pI observed between each band on the IPG strips stained with Coomassie blue matched the calculated pI differences between βI-, βIVb-, and βIII-tubulin and between Kα1- and α6-tubulin (Table 2). The minor band in A549-T12 matched the expected position for βIII-tubulin, and the major ad-

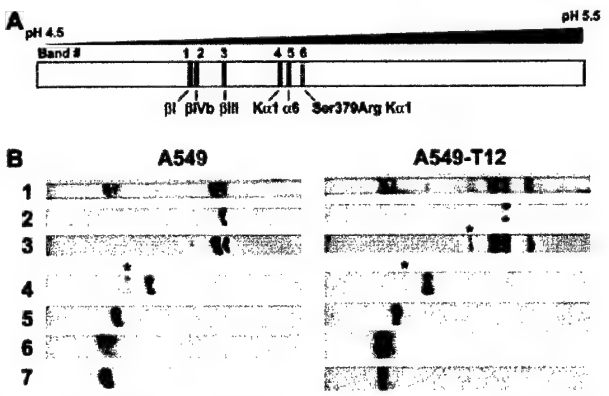

FIGURE 2: IEF-based analysis of tubulin isotypes in A549 and A549-T12 cells. (A) Schematic representation of an IPG strip (pH 4.5−5.5). Predicted positions of tubulin isotypes present in A549 cells are depicted as vertical black bands. A gray band indicates the predicted position of the mutant Kα1-tubulin present in A549-T12 cells. Bands were numbered from the most acidic to the most basic pI value. (B) Tubulin isotypes present in Taxol-stabilized microtubule pellets from A549 (left) or A549-T12 (right) were separated by IEF on 18 cm IPG strips and electrotransferred to nitrocellulose membranes: lane 1, IPG strips stained with Coomassie blue after electrotransfer; lane 2, blots probed with anti-α6-tubulin antibody; lane 3, blots probed with anti-Kα1-tubulin antibody; lane 4, blots probed with anti-βIII-tubulin antibody; lane 5, blots probed with anti-βIV-tubulin antibody; lane 6, blots probed with anti-βI-tubulin antibody; lane 7, blots probed with anti-βII antibody. Asterisks denote minor acidic isoforms (see the Results).

Table 2: pI Differences between Tubulins

ΔpI	calculated	measured[a]
α6 − Kα1	+0.020	+0.017
mutant Kα1 − Kα1	+0.040	+0.044
βIII − βI	+0.050	+0.055
βIVb − βI	+0.010	+0.011
glu Kα1 − Kα1	−0.030	−0.033
glu βIII − βIII	−0.030	−0.027

[a] Based on distances between bands (linear pH gradient).

Table 3: Analysis of Tryptic Digests from IEF Gel Bands by MALDI-TOF Mass Spectrometry

IEF gel piece[a]	tubulin isotype-specific peptide[b]	measured mass of peptide (Da)	calculated mass of peptide (Da)	Δmass (Da)	protein determination[c]
band 1	$_{47}$ISVYYNEATGGK$_{58}$	1300.7	1300.6	−0.1	βI-tubulin
	$_{283}$ALTVPELTQQVFDAK$_{297}$	1658.6	1658.9	0.3	55% sequence coverage
	$_{363}$MAVTFIGNSTAIQELFK$_{379}$	1869.2	1869.0	−0.2	
band 2	$_{63}$AVLVDLEPGTMDSVR$_{77}$	1600.7	1600.8	0.1	βIVb-tubulin
	$_{20}$FWEVISDEHGIDPTGTYHGDSDLQLER$_{46}$	3115.6	3115.4	−0.2	49% sequence coverage
band 3	$_{155}$VREEYPDR$_{162}$	1062.7	1062.5	−0.2	βIII-tubulin
	$_{310}$YLTVATVFR$_{318}$	1068.8	1068.6	−0.2	42% sequence coverage
	$_{47}$ISVYYNEASSHK$_{58}$	1396.6	1396.7	0.1	
	$_{363}$MSSTFIGNSTAIQELFK$_{379}$	1872.6	1872.0	−0.6	
	$_{217}$LATPTYGDLNHLVSATMSGVTTSLR$_{241}$	2604.7	2604.3	−0.4	
	$_{217}$LATPTYGDLNHLVSATMSGVTTSLR$_{241}$ (MSO)	2620.5	2620.3	−0.2	
	$_{20}$FWEVISDEHGIDPSGNYVGDSDLQLER$_{46}$	3076.8	3076.4	−0.4	
band 4	$_{340}$SIQFVDWCPTGFK$_{352}$	1527.5	1527.7	0.2	Kα1-tubulin
	$_{281}$AYHEQLSVADITNACFEPANQMVK$_{304}$ *(MSO)*	2694.0	2694.2	−0.2	60% sequence coverage
	$_{423}$EDMAALEKDYEEVGVDSVEGEGEEEGEEY$_{451}$[d]	3235.4	3235.3	−0.1	
band 5	$_{431}$DYEEVGADSADGEDEGEEY$_{449}$[d]	2077.8	2077.7	−0.1	α6-tubulin
	$_{281}$AYHEQLTVAEITNACFEPANQMVK$_{304}$ (MSO)	2706.5	2706.3	−0.2	56% sequence coverage
	$_{423}$EDMAALEKDYEEVGADSADGEDEGEEY$_{449}$[d]	2964.9	2965.1	0.2	

[a] IEF gel pieces were numbered from the most acidic to the most basic protein band (see Figure 2A). [b] Identified peptides and combinations of peptides specific for a tubulin isotype. MSO represents oxidized methionine. [c] Sequence coverage was calculated on the basis of all the matched tryptic peptides, i.e., tubulin isotype-specific and shared peptides detected. [d] Detection of these peptides was greatly improved in the negative mode.

ditional band, with the most basic pI, matched the position of Kα1-tubulin harboring the Ser379Arg mutation (*20*). The Coomassie blue-stained IPG strips shown in Figure 2 (lane 1) were stained after electrotransfer onto nitrocellulose membranes, indicating that only a small portion of the proteins in each band was effectively transferred.

Validation of Tubulin Isotype Positions on IPG Strips by MALDI-TOF Mass Spectrometry. The position of each tubulin band on the IPG strips was definitively assigned by performing in-gel trypsin digestion followed by analysis of the respective tryptic digests by MALDI-TOF MS (Table 3). The major tubulin species were assigned numbers from 1 to 6 from the most to the least acidic tubulin, i.e., from βI-tubulin to the mutant Kα1-tubulin present in A549-T12 microtubules (Figure 2A). After tryptic digestion of each excised band, mass analysis detected several isotype-specific tryptic peptides that confirmed the assignment of each band to an individual tubulin isotype class based on the sequence-derived pI values (see above). For band 6, corresponding to the mutant Kα1-tubulin, the tryptic peptide profile was identical to the wild-type Kα1-tubulin peptide profile, except that the ion with an *m/z* ratio of 1808.5, corresponding to the peptide 374−390, was not detected, whereas an additional ion with an *m/z* ratio of 1203.5 was present (data not shown). The Ser379Arg replacement in the mutant Kα1-tubulin introduces an additional trypsin cleavage site in the wild-type peptide 374−390 (*m/z* ratio of 1808.1), resulting in two new tryptic peptides, peptide 374−379 (*m/z* ratio of 691.9) that was not detected and peptide 380−390 (*m/z* ratio of 1203.3) that was detected.

Probing of IEF Western Blots with Tubulin Isotype-Specific Antibodies. A polyclonal anti-α6-tubulin antibody recognized one band at the position corresponding to α6-tubulin (Figure 2B, lane 2). Our anti-Kα1-tubulin antibody was apparently also reacting with the α6-tubulin band and labeled the band corresponding to the mutant Kα1-tubulin from A549-T12 (lane 3). A minor α-tubulin species, having a pI more acidic than that of Kα1-tubulin, was also labeled with this antibody and corresponded most likely to monoglutamylated Kα1-tubulin (Table 2) (*14*). The anti-βIII-, anti-βIV-, and anti-βI-tubulin antibodies (Figure 2B, lanes 4−6, respectively) were remarkably specific, each of them labeling only the respective band for βIII-, βIV-, and βI-tubulin. In the case of βIII-tubulin, a second minor band was labeled and could correspond to either monoglutamylated or phosphorylated βIII-tubulin (Table 2; see the Discussion). The anti-βIV-tubulin antibody labeled one band corresponding to βIVb-tubulin. No labeling occurred at positions expected for either of the two possible βIVa-tubulin isotype sequences (Table 1). As expected, the anti-βII-tubulin antibody and the anti-βI-tubulin antibody labeled the same band because these two tubulin isotypes have the same pI value (Figure 2B, lane 7). Because of the previously mentioned potential cross-reactivity of this antibody with βI-tubulin (*21*) and the reported presence of βII-tubulin mRNA in these cells (*13*), we further examined the reactivity of this antibody (see below). The fact that none of the nine βII-tubulin-specific peptides were found in the tryptic digest of the βI-tubulin band (Table 3) strongly suggested that the anti-βII-tubulin antibody was cross-reacting with βI-tubulin.

Relative Quantitation of Tubulin Isotypes in A549 and A549-T12 Cells. We evaluated the amount of total α-tubulin and total β-tubulin by Western blot analysis after complete transfer of tubulin from a SDS−PAGE gel loaded with the different fractions presented in Figure 1. Using a standard curve with bovine brain tubulin and a pan α- or a pan β-tubulin antibody, we obtained an α:β tubulin ratio of 1.1 ± 0.12. This ratio is close to the theoretical ratio of 1, considering that microtubules are formed of tubulin α/β heterodimers. However, the same evaluation of total α-tubulin and total β-tubulin by Western blot analysis, after transferring IEF gels to nitrocellulose, was not possible because, as mentioned above, only a portion of the tubulin contained in each band transferred to the membrane.

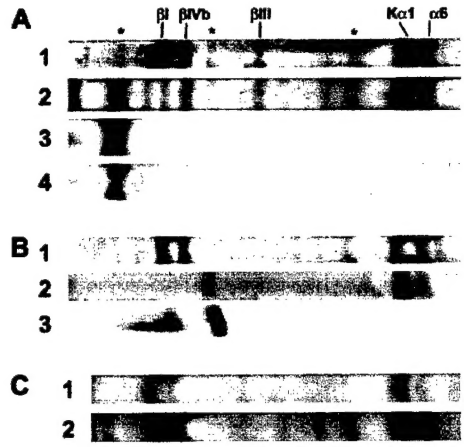

FIGURE 3: Tubulin isotypes in human cancer cell lines. Tubulin was isolated from parental and drug-resistant cell lines and analyzed by IEF. (A) Lane 1, A549 tubulin isotypes stained with Coomassie blue; lane 2, A549·EpoB40 tubulin isotypes stained with Coomassie blue; lane 3, A549·EpoB40 tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti-βI-tubulin antibody; lane 4, A549·EpoB40 tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti βII-tubulin antibody. Asterisks denote minor acidic isoforms. (B) Lane 1, MDA-MB-231 tubulin isotypes stained with Coomassie blue; lane 2, MDA-MB-231·K20T tubulin isotypes stained with Coomassie blue; lane 3, MDA-MB-231·K20T tubulin isotypes blotted on a nitrocellulose membrane that was probed with anti-βI-tubulin antibody. (C) CA46 (lane 1) and HeLa (lane2) tubulin isoforms were stained with Coomassie blue.

Therefore, we stained the IEF gels with Coomassie blue and quantified each tubulin band, and when we summed all the signals from the α-tubulin bands or all the signals from β-tubulin bands, the resulting ratio of total α-tubulin to total β-tubulin was greater than 1 (1.4 ± 0.45 for A549 1.5 ± 0.33 for A549-T12). This ratio was dependent on tubulin concentration, and the ratio decreased to 1.1 when the IEF gels were loaded with a larger amount (>200 μg of protein) of tubulin (see Figure 3A, lane 1). When Fast green was used as the stain in place of Coomassie blue, the same differential staining between α- and β-tubulin was observed (data not shown). It would appear that this excess of α-tubulin over β-tubulin is only apparent and is due to differential staining of α- and β-tubulin by Coomassie blue. For this reason, on Coomassie blue-stained IEF gels, the relative amount of a particular α-tubulin or a particular β-tubulin isotype was calculated as a percentage of the total α-tubulin or total β-tubulin, respectively. From at least three independent measurements, βI- and βIVb-tubulin in A549 microtubules represented 66 ± 10.3 and 29 ± 1.2% of total β-tubulin, respectively. In most samples, βIII could not be detected by Coomassie blue staining except when a larger amount of protein was loaded on the IEF gel (Figure 3A, lane 1). Kα1- and α6-tubulin in A549 represented 66 ± 1.4 and 34 ± 1.3% of the total α-tubulin, respectively. In A549-T12, βI-, βIVb-, and βIII-tubulin represented 56 ± 5.0, 38 ± 4.0, and 6 ± 4.0% of the total β-tubulin, respectively. Kα1-tubulin, α6-tubulin, and the mutant Kα1-tubulin represented 41 ± 3.6, 28 ± 6.0, and 30 ± 2.4% of the total α-tubulin, respectively. These relative percentages of the different tubulin isotypes were similar regardless of the amount of protein loaded on the IEF gel or whether Coomassie blue or Fast green was used as a stain.

Expression of Tubulin Isotypes and of βI-Tubulin Mutations in Cell Lines. We have examined the tubulin isotype composition in other cell lines. Some of these cell lines were resistant to Taxol or epothilones and harbored pI-altering mutations in βI-tubulin. The A549-derived epothilone B-resistant cell line, A549·EpoB40, has a βIGln292Glu mutation (*5*), and we observed the expected shift of βI-tubulin to a more acidic pI with no change in the pIs of other tubulins. However, an apparent decrease in the amount of βIVb-tubulin was noted (Figure 3A, lane 2). Western blotting with the anti-βI-tubulin antibody confirmed that the βI-tubulin shifted to a more acidic pI and demonstrated that only the mutant βI-tubulin, but not the wild type, was expressed in this cell line (lane 3; see the Discussion). When the anti-βII-tubulin antibody was used, the same labeling pattern was obtained as with the anti-βI-tubulin antibody. Importantly, there was no labeling at the position corresponding to βII-tubulin or wild-type βI-tubulin, confirming the cross-reactivity of the anti-βII-tubulin antibody with βI-tubulin (lane 4). The tubulin expression profile in MDA-MB-231 breast cancer cells was not significantly different from the A549 tubulin profile (Figure 3B, lane 1). The MDA-MB-231.K20T cell line has a βIGlu198Gly mutation (*22*), and we observed the expected shift of βI-tubulin to a more basic pI (lane 2). Western blotting with the anti-βI-tubulin antibody confirmed that the βI-tubulin shifted to a more basic pI and demonstrated that most of the βI-tubulin consisted of mutant protein with possibly a trace of wild-type protein (lane 3; see the Discussion). The same labeling pattern was observed with the anti-βII antibody (data not shown). Moreover, in this resistant cell line, βIVb-tubulin, when observed, appeared only as a faint band by Coomassie blue staining compared to the parental cell line. These observations were in agreement with RT-PCR quantitative results on this cell line.[2] In the Burkitt's lymphoma cell line CA46, we observed only two major tubulins, βI- and Kα1-tubulin, and two minor tubulins, βIVb- and α6-tubulin (Figure 3C, lane 1). In HeLa cells, the tubulin profile was very similar to A549 and MDA-MB-231 profiles except that there was an apparent increase in the level of monoglutamylated Kα1 and possibly monoglutamylated βI-tubulin (lane 2).

DISCUSSION

Alterations in tubulin isotype expression and introduction of point mutations into one or both alleles of specific tubulin genes have been associated with resistance to microtubule-stabilizing agents. RT-PCR analysis, although useful for RNA quantitation and sequencing, does not provide insight into the levels of protein expression. With the advent of proteomic-based approaches, it becomes clear that there can be a discrepancy between mRNA transcripts and corresponding protein levels (*23*, *24*).

The isolation of tubulin from cell extracts using Taxol-driven polymerization coupled to high-resolution IEF enabled us to easily visualize and analyze the different tubulin isoforms present in cancer cell lines. We confirmed the presence of the tubulin isotypes in A549 and MDA-MB-231 cell lines identified previously by MALDI-TOF mass spectrometry analysis of C-terminal tubulin peptides (*14*).

[2] K. Wiesen, unpublished observations.

Because the C-terminal sequence obtained previously by MS/MS for α*-tubulin (*14*) matches the C-terminal sequence of the recently described human α6-tubulin, and because in the study presented here a protein having the pI value predicted for human α6-tubulin was detected and confirmed as being α6-tubulin by peptide mapping, we consider them to be identical. An increase in the level of βIII-tubulin was detected in A549-T12 cells compared to the parental A549 cells, which is in agreement with previous immunofluorescence studies (*13*). A minor βIII-tubulin species that corresponded most likely to monoglutamylated or phosphorylated βIII-tubulin was also detected. Only monoglutamylated βI-tubulin, identified previously by MALDI-TOF mass spectrometry analysis of C-terminal tubulin peptides (*14*), was not readily detected by Western blotting (*14*). In all the IPG strips stained with Coomassie blue, we generally observed a barely detectable band at the position of monoglutamylated βI-tubulin, whereas a band at the position of monoglutamylated Kα1-tubulin was clearly visible and was detected by the anti-Kα1-tubulin antibody. Non-neuronal polyglutamylases generate these isoforms (*25*), but the process that limits the glutamylation to only one residue is unclear. The higher percentage of stable microtubules in the brain may favor extensive polyglutamylation, and conversely, the greater dynamicity of cancer cell microtubules (*26*) may limit polyglutamylase activity. Noticeably, known posttranslational modifications of tubulin are predicted to induce a shift in pI values. This is exemplified with the highly modified brain tubulin that appears as multiple bands on IPG strips and gels (*17*, *18*).

In our previous approach, C-terminal peptides were not separated prior to MALDI-TOF mass spectrometry analysis (*14*). βIII-Tubulin was not detected, and βIVb-tubulin represented a very small amount of C-terminal peptide. In this case, ions for minor tubulin species such as βIII-tubulin or βIVb-tubulin could have been suppressed by the presence of the more highly abundant tubulin C-terminal peptides and/or because of differential ionization efficiencies of the various peptides. These technical limitations are overcome in the present isoelectrofocusing/mass spectrometry approach where the total amount of each tubulin isotype is analyzed directly, giving a better appreciation of isotype ratios. Our quantitation of Coomassie blue-stained IEF gels is still only relative. Nevertheless, when LC−MS was used to analyze the same Taxol-stabilized microtubule pellet from A549 and the deconvoluted mass peaks corresponding to each tubulin isotype were integrated, similar relative percentages were obtained.[3] Interestingly, the ratios of Kα1-tubulin to βI-tubulin and α6-tubulin to βIVb-tubulin are close to 1 in the cell lines that have been examined so far. In CA46 Burkitt's lymphoma, α6- and βIVb-tubulin are both minor species. This trend suggests that there could be a specific pairing of a particular α-tubulin isotype with a particular β-tubulin isotype, and we plan to investigate the possibility of such α/β subunit sorting.

The most striking difference between our analysis of tubulin isotype expression at the protein level (ref *14* and this study) and previous studies at the mRNA level (*13*) is the absence of detectable βIVa- and βII-tubulin expression in both sensitive and resistant cell lines. In A549, βIII-, βII-, βIVa-, and βIVb-tubulin mRNA were present at low and similar levels (RT-PCR tubulin isotype:β2-microglobin ratio of ~0.15) that were approximately 10 times lower than that of βI-tubulin mRNA (RT-PCR ratio of ~1.5). A 2-fold increase in βIII-, βII-, and βIVa-tubulin mRNA levels was observed in A549-T12 (*13*). In agreement with the results presented here, both βII- and βIVa-tubulin isotypes could not be detected by MALDI-TOF mass spectrometry of C-terminal peptides derived from total A549 cell extracts (*14*). The reasons for the discrepancy between tubulin isotype mRNA and protein expression levels are not clear, but such differences have been observed for other proteins (*23*, *24*). Since our data established that tubulin was quantitatively recovered from cells by Taxol-driven polymerization (Figure 1), either there is an unknown bias in the RT-PCR results or there is a still unknown silencing mechanism of βII- and βIVa-tubulin mRNA translation. Increased βIII-tubulin expression levels have been repeatedly confirmed at the protein level using a variety of methods in cell lines resistant to Taxol (*13*, *27*−*30*). Together with the study presented here, these observations strongly suggest that only βIII-tubulin levels correlate with sensitivity toward Taxol (*13*, *27*−*30*) and that the regulation of β-tubulin expression is even more complex than the autoregulatory mechanism described by Cleveland and colleagues (*9*).

Mutations that produce a change in charge are likely to have a significant effect on protein function and/or drug binding. In the case of tubulin, we and others have obtained such mutations in cell lines selected for resistance to microtubule-stabilizing drugs (*5*−*8*). When analyzed by two-dimensional gel electrophoresis, the wide pH range used in the first dimension resulted in a poor resolution of the spots corresponding to wild-type and mutant proteins and prevented quantitation. Like the expression of a particular isotype, the detection of wild-type and mutant mRNA of a specific tubulin does not indicate that it is translated and, if so, at what ratio. Most likely, mutations affect only one allele (*8*), and it is expected that both tubulin isoforms would be produced in equal amounts. We found that situation in A549-T12, where the percentage of wild-type Kα1-tubulin decreased at the expense of mutant Kα1-tubulin (~30% of the total α-tubulin). But in two cell lines, MDA-MB-231·K20T and A549·EpoB40, the wild-type βI-tubulin mRNA was not detected by RT-PCR (*5*, *22*) and only the mutant protein was detected in these cells. The heterozygous status would need to be checked at the DNA level, and if confirmed, it would imply a silencing mechanism of the wild-type allele (*8*). If a mutation in tubulin plays a role in resistance to microtubule-interacting agents, the ratio of mutant tubulin to wild-type tubulin in a cell should influence the degree of resistance. In fact, when equal amounts of wild-type and mutant tubulin are expressed, the level of resistance to Taxol tends to be lower than when only the mutant tubulin is expressed (*5*, *8*, *13*).

The anti-β-tubulin isotype monoclonal antibodies produced and characterized by R. F. Ludueña and co-workers (*31*−*33*) represent valuable tools that have helped to unravel the functional significance of the different β-tubulin isotypes. No cross-reactivity of the anti-βI-, anti-βIII-, and anti-βIV-tubulin antibodies was observed in this study. This is contrary

[3] P. Verdier-Pinard, manuscript in preparation.

to what has been published for nondenaturing immunoaffinity isolation of α/β-tubulin heterodimers enriched in βI-, βII-, βIII-, or βIV-tubulin isotypes (*21*, *31*, *34*, *35*). Therefore, the presence of contaminating β-tubulin isotypes of a different class in these fractions is more likely due to heterodimer−heterodimer interactions in residual small oligomers than to cross-reactivity of these antibodies with multiple isotypes. However, extensive cross-reactivity of the anti-βII-tubulin antibody with βI-tubulin was confirmed. The peptide EGEEDEA that was used to generate the anti-βII-tubulin antibody is from the C-terminal sequence of chicken βII-tubulin (EEGEDEA in humans; see Table 1) (*21*). The human βI-tubulin C-terminus contains the sequence GEEAEEEA, so it is possible that the anti-βII-tubulin recognizes the GEE sequence present in the βI-tubulin C-terminus. Recently, Arai et al. (*36*) described an anti-βII-tubulin polyclonal antibody that specifically recognized the EEEEGED sequence of the human βII-tubulin C-terminus and did not cross-react with C-terminal peptides of other β-tubulin isotypes. The peptide EAEEEVA that is common to βIVa and βIVb was used to generate the anti-βIV-tubulin antibody (*34*). Thus, both isotypes, with clearly distinct pIs, can be labeled with this antibody. However, we have observed only the presence of βIVb-tubulin in the cell lines examined so far.

There are fewer α-tubulin isotype-specific antibodies available (*37*). A rabbit polyclonal antibody against human α6-tubulin, made in our laboratory by S. Rao, did not cross-react with Kα1-tubulin. However, the anti-Kα1-tubulin polyclonal antibody cross-reacted with α6-tubulin. Further testing of these antibodies on samples containing different α-tubulin isotypes will permit a more extensive definition of their specificity. Altogether, our approach provides a method for screening the specificity of antibodies directed against tubulin isotypes and establishes that the anti-βI-, anti-βIII-, anti-βIV-tubulin antibodies are highly specific.

A similar analysis of tubulin isotypes at the protein level in human tumor biopsies would provide an alternative to RT-PCR for investigating the clinical relevance of differential expression of tubulin isotypes in relation to the responsiveness of patients to microtubule-interacting drugs. Recent clinical data from non-small cell lung cancer patients indicated that both βIII-tubulin mRNA levels (*38*) and βIII-tubulin protein levels (*39*) were higher in tumors that did not respond to taxane-based treatment. Finally, the microtubule cytoskeleton appears to be increasingly involved in several neurodegenerative diseases (*40*−*45*), and comparative analysis of tubulin protein expression with normal tissue would provide greater insight into these pathologies.

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